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Study of *in vitro* conditions modulating expression of MN/CA IX protein in human cell lines derived from cervical carcinoma*

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In an effort to better understand the biological significance of MN/CA IX human tumor-associated protein, we have investigated its expression in human cervical carcinoma cell lines *in vitro*. SiHa cells that naturally express MN/CA IX were used as a model for expression study at the protein level. In addition, we have transfected *MN/CA9* gene-negative but transcription-competent C33A cells with a plasmid carrying CAT reporter gene under a control of *MN/CA9* promoter. By this way, we have generated a stable cell line C33A/MNP-CAT that was employed in analysis of *MN/CA9* regulation at the level of promoter activity as estimated by CAT protein abundance. For the purpose of our study, we have chosen experimental conditions relevant to growth characteristics and phenotypic features of malignantly transformed cells. Both the level of MN/CA IX protein and the gene promoter activity were found to be substantially elevated either in culture of high density or when the adherent carcinoma cells grew in suspension, but were not markedly affected by diminished serum concentration and in the cell cycle progression. These observations support the involvement of MN/CA IX protein in aberrant cell-cell and cell-matrix interactions that facilitate loss of contact inhibition and anchorage independence of cancer cells.

Key words: MN/CA IX protein, cervical carcinoma cell lines, contact inhibition, anchorage independence.

MN/CA IX is a transmembrane glycoprotein of 58/54 kDa, detected at the surface of various carcinoma cells both *in vitro* and *in vivo* [11, 23]. It has gained attention as an emerging biomarker of several types of human tumors, namely carcinomas of the cervix uteri, kidney, esophagus, colon, and lung [5—8, 17, 20, 22]. Presence of MN/CA IX in cancer tissues and absence in their normal counterparts indicate its possible role in carcinogenesis. In culture, MN/CA IX protein expression is positively regulated by increasing density of HeLa cells and correlates with the tumorigenic phenotype of hybrid cells constructed by fusion of HeLa and normal human fibroblasts [23].

Cloning and sequencing of both *MN/CA9* cDNA and gene allowed a prediction of a structural composition of MN/CA IX protein [9, 10] and revealed that it belongs to a family of carbonic anhydrases (CA), whose members catalyze reversible hydration of carbon dioxide to carbonic acid. Via this activity, CAs participate in a variety of biological processes [18]. The CA domain with conserved enzyme active site encompasses almost whole extracellular part of MN/CA IX. It is flanked by a small N-terminal region homologous to proteoglycans, and a C-terminal extension composed of a transmembrane anchor and a short intracytoplasmic tail [9]. On the basis of the cDNA sequence homology it was recently shown that MN/CA IX is identical with G250 renal cell carcinoma-specific antigen [21].

Introduction of *MN/CA9* cDNA into NIH 3T3 cells results in their morphological transformation and increased proliferation [10]. This is in agreement with observation in colorectal neoplasia that MN/CA IX protein co-occurs with Ki-67 cell proliferation marker in areas of high proliferative activity [17].

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Based on the expression pattern, MN/CA IX appears to participate in intercellular communication and in control of cell proliferation, but its precise function is still unclear. One of the approaches leading to better understanding of biological significance of MN/CA IX is the analysis of *in vitro* conditions regulating its biosynthesis. In this paper, we present a study of MN/CA IX expression at the level of promoter activity and protein synthesis in human cell lines SiHa and C33A derived from carcinoma of the cervix uteri. We found that both the amount of MN/CA IX protein and the promoter activity are remarkably elevated in cells cultivated either at high density or in suspension culture. On the other hand, level of MN/CA IX was not markedly affected by serum deprivation and did not change during the cell cycle in the synchronized SiHa cells. These data support the involvement of MN/CA IX in aberrant cell-cell and cell-substrate signaling.

Material and methods

Cell culture. Human cervical carcinoma cell lines SiHa and C33A were maintained in Dulbecco's MEM (BioWhittaker, USA) supplemented with 10% FCS (GibcoBRL, USA) and 40 μg/ml gentamicine (Lek, Slovenia). For the analysis of density-dependence, the cells were plated at different densities (D) so that D1 corresponded to 10^4 cells/cm^2, while the higher Ds were the multiplicates of this cell number. After 72 hrs of incubation, the cells were extracted for the analysis of MN/CA IX protein level and promoter activity, respectively. For the serum starvation, the cells were plated at D6 in DMEM with 10% FCS and incubated for 24 hrs. Then the medium was removed, the cells were washed twice with PBS, and DMEM containing 0.1% FCS was added. After 48 and 72 hrs, respectively, the cells were subjected to extraction. Experiments with suspension cultures were made in Petri dishes coated with 10 mg/ml ethanol solution of polyHEMA (Sigma, USA) as recommended by manufacturer. After 72 hrs of cultivation, the cells were collected and extracted for further analyses.

Western blotting. Cells were extracted with RIPA buffer (7.5 mmol phosphate buffer pH 7.2, 140 mmol NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mmol PMSF and 10 μmol Trasylol) for 3 min at 4°C. Total protein concentrations were determined in all extracts using BCA kit (Pierce, USA). Samples containing equal amounts of total proteins were resolved in 10% SDS-PAGE and transferred to Hybond ECL membrane (Amersham, UK). The membrane was blocked in TBS (50 mmol Tris-HCl pH 7.5, 150 mmol NaCl) containing 5% non-fat milk, incubated with MN/CA IX-specific monoclonal antibody M75 [11], washed with TBS, incubated with horseradish peroxidase-conjugated swine anti-mouse antibodies and subjected to ECL detection (Amersham, UK).

Construction of pBL6-MNP-CAT plasmid. MN/CA9 promoter (MNP) fragment corresponding to genomic region -172/+31 with respect to transcription initiation site was derived from Bd3 genomic clone in pBluescriptII KS$^+$ [9] by unidirectional nested deletions and subcloned to pBL6CAT vector [2]. Correct insertion of the MNP fragment was verified by sequencing of both ends using T7 sequencing kit (Pharmacia, Sweden).

Generation of C33A/MNP-CAT cell line. C33A cells were co-transfected with 10 μg of pBL6-MNP-CAT and 1 μg pSV2neo plasmids by a calcium phosphate precipitation method using Mammalian Transfection Kit (Stratagene, USA). Transfected cells were subjected to selection with 700 μg/ml G418. Selected clones were isolated, expanded and tested for CAT activity. One of several clones continuously producing detectable level of CAT was used for the present study.

Determination of CAT level and activity. Level of CAT protein synthesized in C33A/MNP-CAT cells was measured using CAT ELISA as recommended by the manufacturer (Boehringer Mannheim, FRG). The cells were lysed in CAT ELISA lysis buffer and total protein concentrations in all lysates were determined by BCA kit (Pierce, USA). Samples containing equal amounts of total proteins were used for the analysis. In some experiments, chloramphenicol acetyltranferase activity was detected by CAT assay followed by thin layer chromatography [1].

Slot blot hybridization. Synchronized SiHa cells harvested at different time points during the cells cycle were used for extraction of total cellular RNA by acid guanidinium-thiocyanate phenol-chloroform method [3]. 5 μg of RNA from each sample was diluted in MOPS buffer, denatured by heating at 65°C for 5 min and applied to a Hybond N$^+$ membrane (Amersham, U.K.) using HYBRI-SLOT^{TM} apparatus (GibcoBRL, USA). Membrane was probed with *MN/CA9* cDNA excised from pBluescript and labeled with [α-^{32}P]-dCTP using the Megaprime DNA Labeling System (Amersham, U.K.). Prehybridization, hybridization and washing under stringent conditions were performed according to [1]. Slot blot was analyzed by autoradiography.

Immunofluorescence. Cells grown on glass cover slips were fixed with methanol at -20°C for 5 min, washed with PBS, incubated for 30 min in PBS containing 1% BSA and treated for 1 h at 37°C with the M75 monoclonal antibody in hybridoma medium [11]. After washing, FITC-conjugated swine anti-mouse IgG was added for 1 hr. Then the samples were washed with PBS, mounted in anti-bleach medium (PBS containing glycerol, formaldehyde and citifluor in ratio 9:1:1), and examined with a Nikon fluorescence microscope.

Cell synchronization. Asynchronous SiHa cells grown for 24 hrs were treated by 2 mmol thymidine for 16 hrs. Then the cells were washed by PBS and incubated in medium without thymidine. After 7 hrs, the thymidine block was repeated once. Following release of the second block, the population enriched

for G1/S cells entered the cycle. At the time points of 0, 5, 9, 12, 16, and 24 hrs, the cells were harvested for the analysis of protein and RNA levels and for monitoring the cell cycle distribution by flow cytometry.

Flow cytometry. Immediately after harvesting, 2 x 10^6 cells were fixed by 70% ethanol at –20°C. Then they were allowed to rehydrate in PBS, treated by 10 mg/ml RNase A (Sigma, USA) and stained with 50 µg/ml propidium iodide (Sigma, USA). The analysis was performed using Becton-Dickinson FACStar flow cytometer. A total of 10,000 cells were analyzed per sample.

Results

Model cell lines. MN/CA IX protein was originally identified in cervical carcinoma cell line HeLa and in tumorigenic hybrids between HeLa and normal human fibroblasts [11, 23]. Later on, it was shown to be expressed in virtually all cervical carcinomas and in the majority of cervical intraepithelial neoplasia, but not in normal cervices [5, 6]. In this study, we have chosen SiHa cells that express MN/CA IX and are derived from advanced cervical cancer, as a suitable model for the analysis of *in vitro* modulation of MN/CA IX protein synthesis.

We have also used a cervical carcinoma cell line C33A that does not express MN/CA IX protein due to the absence of *MN/CA9* gene as revealed by Southern blotting (data not shown). However, in transient transfections this cell line was found to be competent to drive the transcription of chloramphenicol acetyl transferase (CAT) reporter gene from *MN/CA9* promoter (MNP). Based on this fact, we have decided to generate a stable cell line that would continuously produce CAT protein under an MNP control. In those cells, the level of CAT could serve as an indicator of the promoter activation/repression upon various cultivation conditions. Additional advantages of this model were: (1) presence of the transfected MNP-CAT DNA in each cell of the population, resulting in a better detection of weak MNP activity, and (2) the possibility of quantitative evaluation of changes in MNP activity *via* determination of CAT levels. Moreover, employment of *MN/CA9* gene-negative C33A cells allowed us to avoid illegal homologous recombination of MNP with the endogenous sequences.

C33A cells were co-transfected with pBL6-MNP-CAT and pSV2neo plasmids and selected with G418. Drug-resistant colonies were picked, expanded and tested for CAT expression. Several cell lines showed detectable CAT activity (Fig. 1). One of them (Fig. 1, line 3) was used in this study. The results on MNP activity obtained with the help of C33A/MNP-CAT cells were compared to the data on regulation at the MN/CA IX protein level in SiHa cells.

Effect of cell density. One of the first observations related to the *in vitro* expression of MN/CA IX protein was its dependence on HeLa cell density [11]. As shown in Fig. 2a, the cell density had the same effect in SiHa cells. Expression of 58/54 kDa MN/CA IX protein was induced in culture whose cells came into close contact and formed a multilayer, i.e. in a situation when they circumvented contact inhibition. Immunofluorescence labeling of MN/CA IX protein in dense SiHa cells clearly demonstrated that the MN/CA IX protein was synthesized in cells that were not adherent to plastic support, but grew in upper layers that were lacking a direct contact with the support (Fig. 3).

Analysis of CAT protein produced in C33A/MNP-CAT cells cultivated in three different relative densities gave the results corresponding to data obtained in SiHa cells (Fig. 2b, c) and revealed that the effect of density is exerted at the level of transcription initiation. These results were also in accord with the Northern blotting analysis of *MN/CA9* mRNA level in dense versus sparse HeLa cells published previously [10].

Expression in suspension cells. Anchorage independence is a hallmark of malignant transformation *in vitro*. It is due to aberrant signal transduction from extracellular matrix or adjacent

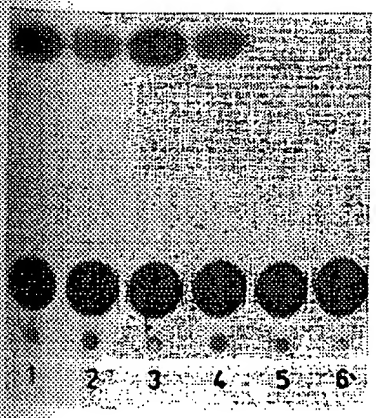


Fig. 1. Analysis of CAT activity in C33A cells co-transfected with pBL6-MNP-CAT and pSV2neo plasmids. The transfected cells were subjected to G418 selection, cloned, expanded and tested by CAT assay and thin layer chromatography. Clonal cell lines synthesizing CAT from *MN/CA9* promoter (1—4), CAT-negative clones (5, 6).

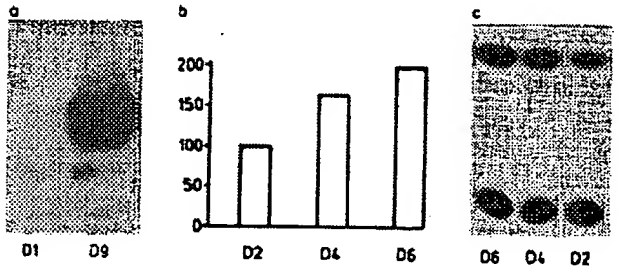


Fig. 2. Cell density effects. MN/CA IX protein of 58/54 kDa was detected in SiHa cells by Western blotting (a). *MN/CA9* promoter activity in C33A/MNP-CAT cells was determined by CAT ELISA (b), and CAT assay followed by thin layer chromatography (c). Cell densities at plating are shown below the samples: D1 corresponds to 10^4 cells/cm^2, Dn is n x 10^4 cells/cm^2. Level of CAT protein in each sample was determined as pg of CAT per 100 µg of total proteins, then the percentage was calculated and shown at y axis.

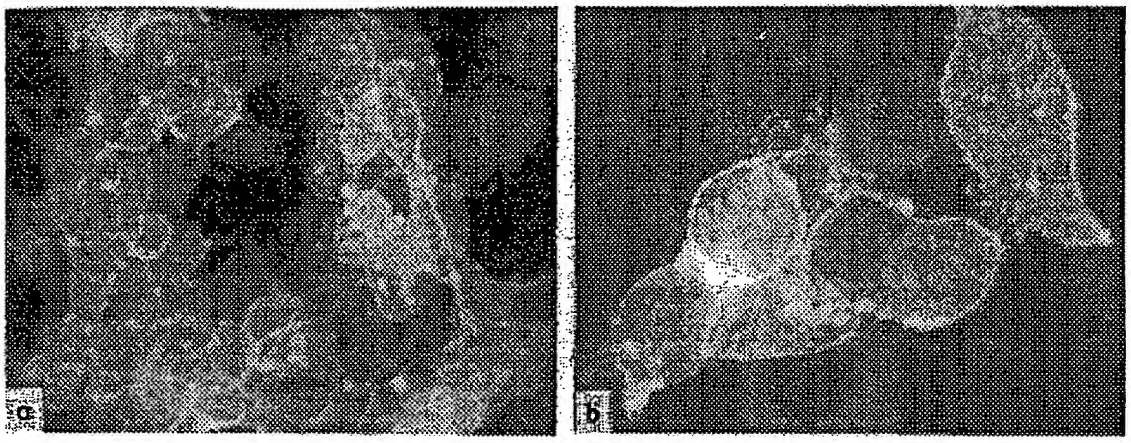
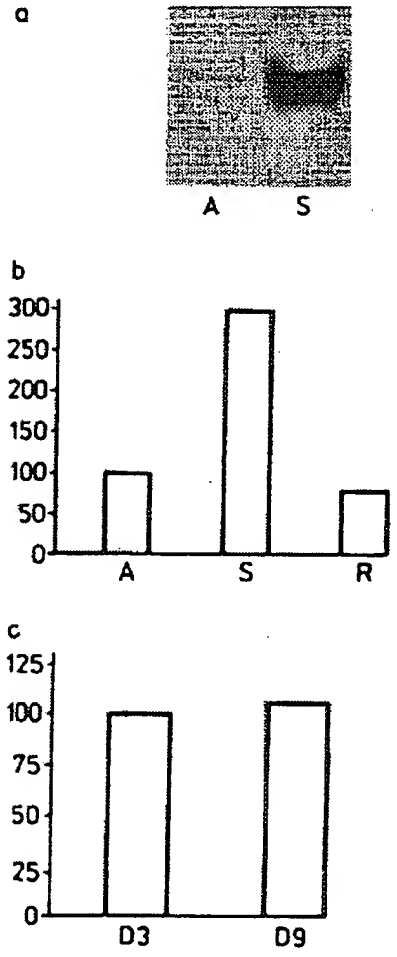


Fig. 3. Immunofluorescence detection of MN/CA IX protein in dense culture of SiHa cells using monoclonal antibody M75. The microscope was focused at the cells in the upper layers that show elevated expression of MN/CA IX protein. Magnification x200 (a), x600 (b).



cells resulting in deregulated expression of numerous genes involved in cell adhesion [16]. This knowledge as well as our findings described above prompted us to analyze whether expression of MN/CA IX is differentially controlled in the cells anchored to the solid support versus cells grown in suspension. Western blotting analysis of SiHa cells revealed that synthesis of MN/CA IX protein is markedly elevated in suspension culture when compared to adherent cells plated at the same density (Fig. 4a). The result corresponded well with the immunofluorescence detection of MN/CA IX protein in multilayer culture of SiHa cells (Fig. 3). Similarly, C33A/MNP-CAT cells grown in suspension exhibited substantially higher level of CAT protein than those grown in adherent culture or after re-adhesion of suspension cells to solid support (Fig. 4b). These data showed that the *MN/CA9* promoter was activated following disruption of cell-support contact and that the activation ceased after renewal of this interaction. The results obtained here indicate a possible role of MN/CA IX in facilitating the anchorage independent growth of carcinoma cells.

Noteworthy, increasing density of C33A/MNP-CAT cells cultivated in suspension did not have any profound effect on the activity of *MN/CA9* promoter (Fig. 4c), in contrast to situation shown above in adherent cells (Fig. 2b). The density-independent expression from *MN/CA9* promoter in this experimental condition was apparently due to the fact that the density signaling was mediated by direct cell-cell contacts whose establishment was eliminated in suspension culture.

Influence of diminished serum concentration. Serum is an important component of cultivation media as a source of

Fig. 4. Adhesion-modulated expression of MN/CA IX protein in SiHa cells (a) and of CAT protein in C33A/MNP-CAT cells (b, c). The cells were grown in adherent culture (A), in suspension (S), or were re-attached for 4 hrs (R) (b). C33A/MNP-CAT cells cultivated in suspension at two different densities. Percentage of CAT was calculated as in Fig.2 and shown at y axis.

growth factors that provide mitogenic and survival signals. They act through different pathways and control expression of a variety of genes [15]. Cells in dense culture have higher serum requirements than the sparse cells grown in the same volume of medium. Thus, it was possible that the higher levels of MN/CA IX in the denser cultures could be attributed to more rapid consumption of serum growth factors. Therefore, we have analyzed an effect of diminished serum concentration on expression of MN/CA IX protein as well as on the gene promoter activity. SiHa cells were either cultivated in medium containing 10% FCS or subjected to serum starvation in medium with 0.1% FCS for 48 hrs and 72 hrs, respectively. In both starvation intervals, the level of MN/CA IX was only barely decreased compared to non-starved control as detected by Western blotting (Fig. 5a). In starved C33A/MNP-CAT cells, activity of MN/CA9 promoter was slightly higher when compared to that in control cells cultivated in 10% serum (Fig. 5b). The minor difference between CAT levels in starved versus control cells roughly corresponded to the difference observed between suspension cells grown in dense versus sparse culture (see Fig. 4c).

The results of these experiments have shown that the expression of MN/CA IX was not markedly affected by decreased level of serum and indicated that the effect of density was true and not mediated indirectly by the depletion of serum growth factors.

Expression of MN/CA IX during the cell cycle. Pattern of MN/CA IX expression *in vitro* and *in vivo*, especially the observed co-occurrence with the proliferation marker Ki-67 in intestinal mucosa [17] indicated possible relationship between the cell cycle progression and the regulation of MN/CA IX. On this basis, we have decided to examine the profile of *MN/CA9* transcription and protein synthesis during the traverse of the cell cycle in SiHa cells that were arrested at early S phase by double thymidine block. Following the block release, the cells were harvested at several time points for both protein and mRNA analyses. The cell cycle distribution of analyzed population in given time points was determined by monitoring the DNA content with a flow cytometer. As shown in Fig. 6, the abundance of mRNA did not fluctuate with the cell cycle. Also the MN/CA IX protein level remained fairly constant. For comparison, we have analyzed C33A/MNP-CAT cell populations enriched for cells in G1, S or G2/M, respectively. Again, we did not find any considerable difference in CAT levels indicating that the promoter activity did not change in the analyzed stages of the cell cycle (Fig. 7). There was one problematic point that has to be taken into account when evaluating the above experiment. The fact that the expression of MN/CA IX was density-dependent did not allow us to use sparse, exponentially growing cells and as a consequence, the synchronization was not so efficient. Despite this drawback, data obtained here provide sufficient evidence for the steady expression of MN/CA IX during the cell cycle.

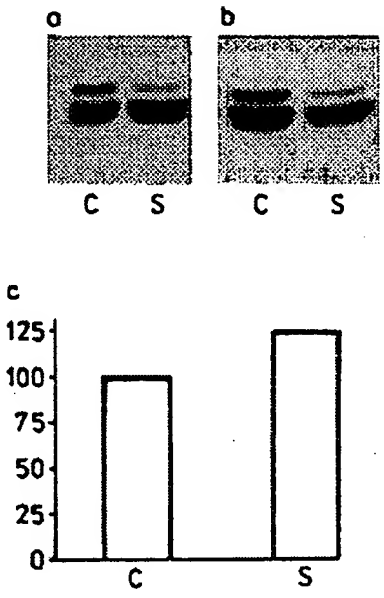


Fig. 5. Synthesis of MN/CA IX protein in SiHa cells (a, b) and MNP transcriptional activity in C33A/MNP-CAT cells (c) grown in diminished serum concentration. Control cells (C) were cultivated in medium with 10% FCS, while the medium of the starved cells (S) contained 0.1% FCS. The cells were harvested after 48 hrs (a) or 72 hrs (b, c) of growth. Percentage of CAT level was calculated as in Fig.2 and shown at y axis.

Discussion

In the process of neoplastic transformation, cells acquire a number of new properties that allow them to circumvent both internal and external control mechanisms [14, 19]. Phenotypic changes of the transformed cells result from alterations in multiple genes or from their deregulated expression that interfere with the normal signal transduction pathways. Expression of human *MN/CA9* gene has been associated with a number of malignancies indicating its possible participation in cell transformation. In the present *in vitro* study using human cervical carcinoma cell lines, we have shown that the *MN/CA9* gene is activated in cells grown at high density and in suspension cultures, respectively, i.e. in situations that are restrictive for growth of normal non-transformed cells.

The main *in vitro* characteristics of cancer cells involve indefinite growth, loss of contact inhibition and gain of anchorage independence [19]. Whereas normal cells cease to proliferate when confluent, transformed cells do not have this property and continue in proliferation after reaching the confluence. Interestingly, expression of *MN/CA9* gene is induced in those tumor cells that overcome the putative point at which normal cells are contact inhibited. As we found here, this induction occurs at the level of transcription initiation and is also mirrored by the protein abundance.

MN/CA IX is an integral transmembrane protein localized at the cell surface and thus, it might play a role in outside-in transduction of density signals. However, the increasing cellular density stimulated the expression of CAT from *MN/CA9* promoter also in C33A cells that do not possess MN/CA

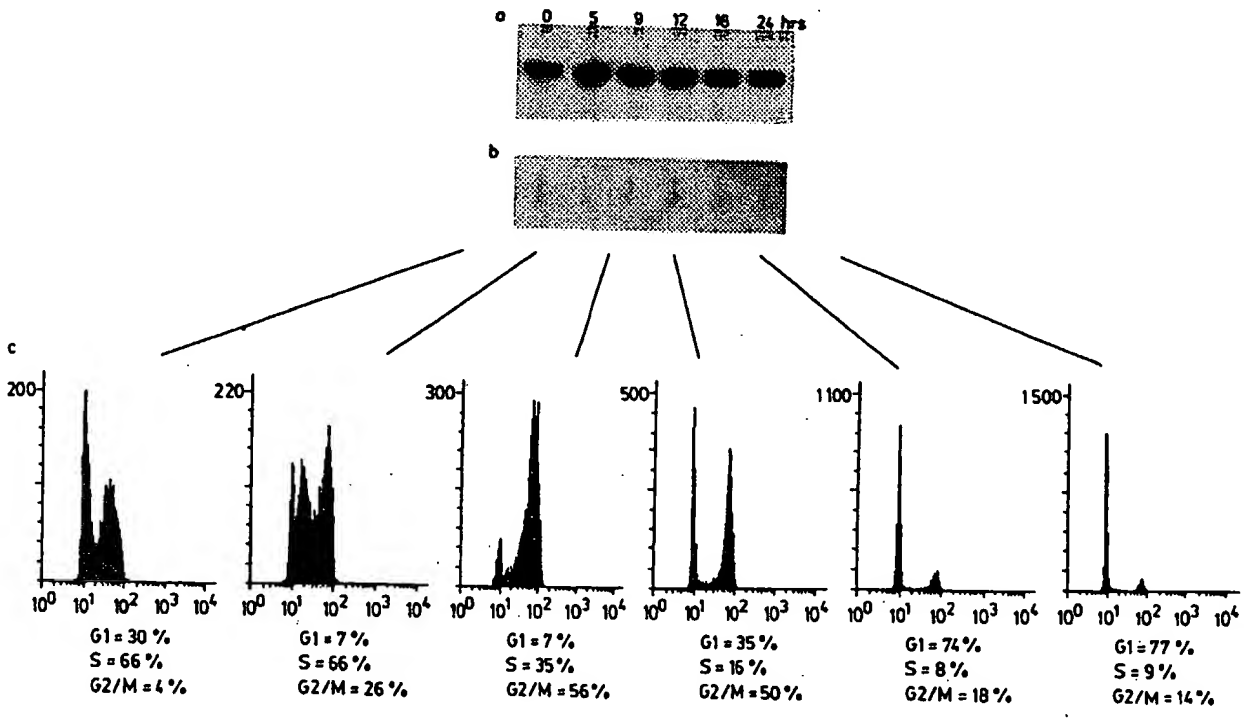


Fig. 6. Cell cycle analysis of synchronized SiHa cells. Expression of MN/CA IX was detected by Western blotting at the protein level (a) and by slot blot hybridization at the mRNA level (b). The cell cycle distribution of SiHa populations harvested at the time points given above the blots was monitored by flow cytometry (c).

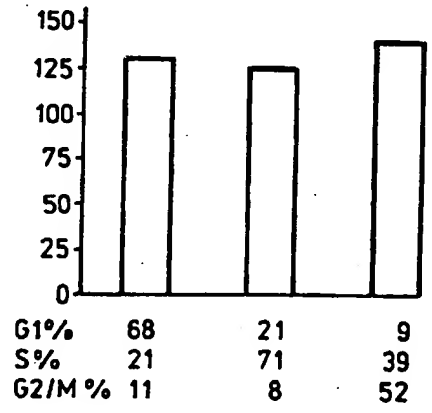


Fig. 7. Synthesis of CAT protein driven by *MN/CA9* promoter in C33A/MNP-CAT populations enriched for G1, S, and G2/M cells, respectively. The cells were synchronized and allowed to traverse the cell cycle. Populations harvested during the cycle were analyzed by flow cytometry and the corresponding samples were subjected to CAT ELISA. Level of CAT protein in each sample was determined as pg of CAT per 100 μg of total proteins, then the percentage was calculated and shown at y axis.

IX protein. Thus, we assume that this protein could rather function as an effector molecule whose expression is activated after the cells come into close contact, with the purpose to facilitate the cell growth beyond the confluence.

Very similar conclusion can be drawn from the observed increase of both *MN/CA9* promoter activity and protein level in carcinoma cells cultivated in suspension. Expression of *MN/CA9* gene is activated following disruption of cell-support contact and is decreased after its re-establishment. The same pattern of the regulation in SiHa (MN/CA IX protein positive cells) and C33A (MN/CA IX protein negative cells) indicates again that MN/CA IX protein is not required for initial phase of loss-of-attachment signal transduction to cell interior. It rather appears to represent the cellular response that either involves signaling to adjacent cells or interferes with the normal cell adherence signals.

On the other hand, serum starvation and cell cycle progression were found to have no significant influence on *MN/CA9* gene expression in the terms of our experimental conditions. It is well known that decreased serum level negatively regulates the expression of certain proliferation- and cell cycle-associated genes, but stimulates the expression of genes that are involved in the differentiation or apoptosis

[4, 14]. However, cancer cells show diminished response to serum factors when compared to their non-transformed counterparts and possess defects in the cell cycle checkpoints [13, 19]. Therefore, we cannot exclude the possibility that more strict conditions of starvation and synchronization or the employment of another cellular model would provide different results. Nevertheless, in view of the present data, MN/CA IX synthesis in carcinoma cells SiHa and C33A/MNP-CAT appears independent of regulatory pathways mediated by serum growth factors and the cell cycle control.

Taken together, association of MN/CA IX expression with increasing density of cervical carcinoma cells as well as with the loss of their adherence supports the involvement of MN/CA IX protein in cell-cell and cell-matrix interactions. Noteworthy, in epithelia of gastrointestinal tract that represent the only non-cancerous tissues expressing MN/CA IX, the protein is localized at the basolateral cell surfaces [12, 17]. It was proposed that this subcellular localization is due to possible functional significance of MN/CA IX in intercellular communication and in cell adhesion to basal membrane. Moreover, MN/CA IX protein was shown to act as an adhesion molecule *in vitro* [24]. On the basis of our knowledge from the past and present studies, it is not inconceivable that the proposed normal physiological role of MN/CA IX in cell adhesion might be converted in transformed cells to abnormal activity disturbing their communication and growth control. Nevertheless, to confirm this assumption, further investigation of MN/CA IX regulation and its functional significance is warranted.

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References

[1] Ausubel, F.M., Brent, R., Kingstone, R.E., More, D.D., Seidman, J.G., Smith, J.A., Struhl, K.: Short Protocols in Molecular Biology. New York, Greene Publishing Associates, Wiley-Interscience 1989.

[2] Boshart, M., Kluppel, M., Schmidt, A., Schutz, G., Luckow, B.: Reporter constructs with low background activity utilizing the CAT gene. Gene, *110*, 1992, 129—130.

[3] Chomczynski, P., Sacchi, N.: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., *162*, 1987, 156—159.

[4] Fuchs, E.: Epidermal differentiation and keratin gene expression. J. Cell. Sci., Suppl., *17*, 1993, 197—208.

[5] Liao, S.Y., Brewer, C., Závada, J., Pastorek, J., Pastoreková, S., Manetta, A., Berman, M., Disaia, P.J., Stanbridge, E.J.: Identification of the MN antigen as a diagnostic biomarker of cervical intraepithelial squamous and glandular neoplasia and cervical carcinomas. Am. J. Pathol., *145*, 1994, 598—609.

[6] Liao, S.Y., Stanbridge, E.J.: Expression of the MN antigen in cervical Papanicolaou smears is an early diagnostic biomarker of cervical dysplasia. Cancer Epidemiol. Biomarker Prev., *5*, 1996, 549—557.

[7] Liao, S.Y., Aurelio, O.N., Jan, K., Závada, J., Stanbridge, E.J.: Identification of the MN/CA9 protein as a reliable diagnostic biomarker of clear cell carcinoma of the kidney. Cancer Res., *57*, 1997, 2827—2831.

[8] McKiernan, J.M., Buttyan, R., Bander, N.H., Stifelman, M.D., Katz, A.E., Chen, M.W., Olsson, C.A., Sawczuk, I.S.: Expression of the tumor-associated gene MN: A potential biomarker for human renal cell carcinoma. Cancer Res., *57*, 1997, 2362—2365.

[9] Opavský, R., Pastoreková, S., Zelník, V., Gibadulinová, A., Stanbridge, E.J., Závada, J., Kettmann, R., Pastorek, J.: Human MN/CA9 gene, a novel member of the carbonic anhydrase family: Structure and exon to protein domain relationships. Genomics, *33*, 1996, 480—487.

[10] Pastorek, J., Pastoreková, S., Callebaut, I., Mornon, J.P., Zelník, V., Opavský, R., Zaťovičová, M., Liao, S., Portetelle, D., Stanbridge, E.J., Závada, J., Burny, A., Kettmann, R.: Cloning and characterization of MN, a human tumor-associated protein with a domain homologous to carbonic anhydrase and putative helix-loop-helix DNA binding segment. Oncogene, *9*, 1994, 2878—2888.

[11] Pastoreková, S., Závadová, Z., Košťál, M., Babušíková, O., Závada, J.: A novel quasi-viral agent MaTu, is a two-component system. Virology, *187*, 1992, 620—626.

[12] Pastoreková, S., Parkkila, S., Parkkila, A.-K., Opavský, R., Zelník, V., Saarnio, J., Pastorek, J.: Carbonic anhydrase IX, MN/CA IX: Analysis of stomach complementary DNA sequence and expression in human and rat alimentary tracts. Gastroenterology, *112*, 1997, 398—408.

[13] Paulovich, A.G., Toczyski, D.P., Hartwell, L.H.: When checkpoints fail. Cell, *88*, 1997, 315—321.

[14] Reichmann, E.: Oncogenes and epithelial cell transformation. Semin. Cancer Biol., *5*, 1994, 157—165.

[15] Rollins, B.J., Stiles, C.D.: Serum-inducible genes. Adv. Cancer Res., *53*, 1—33.

[16] Ruoslahti, E., Öbrink, B.: Common principles in cell adhesion. Exp. Cell Res., *227*, 1996, 1—11.

[17] Saarnio, J., Parkkila, S., Parkkila, A.-K., Haukipuro, K., Pastoreková, S., Pastorek, J., Kairaluoma, M.I., Karttunen, T.J.: Immunohistochemical study of colorectal tumors for expression of a novel transmembrane carbonic anhydrase, MN/CA IX, with potential value as a marker of cell proliferation. Am. J. Pathol., *153*, 1998, 279—285.

[18] Sly, W.S., Hu, P.Y.: Human carbonic anhydrases and carbonic anhydrase deficiencies. Annu. Rev. Biochem., *64*, 1995, 375—401.

[19] Sun, T.-T., Eichner, R., Schermer, A., Cooper, D., Nelson, W.G., Weiss, R.A.: The Transformed Phenotype. I. The Cancer Cell. New York, Col. Spring Harbor 1984, 169—176.

[20] Turner, J.R., Odze, R.D., Crum, C.P., Resnick, M.B.: MN antigen expression in normal, preneoplastic, and neoplastic esophagus: A clinicopathological study of a new cancer-associated biomarker. Hum. Pathol., *28*, 1997, 740—744.

[21] UEMURA, H., KITAGAWA, H., HIRAO, Y., OKAJIMA, E.: Expression of tumor-associated antigen MN/G250 in urologic carcinoma: Potential therapeutic target. J. Urol., *157*, 1997, 377.

[22] VERMYLEN, P., ROUFOSSE, C., NINANE, V., SCULIER, J.P., BURNY, A.: Expression of the MN antigen as a biomarker of lung carcinoma and associated precancerous conditions. Proc. 89th Annual Meeting of the AACR, *39*, 1998, 2280.

[23] ZÁVADA, J., ZÁVADOVÁ, Z., PASTOREKOVÁ, S., ČIAMPOR, F., PASTOREK, J., ZELNÍK, V.: Expression of MaTu-MN protein in human tumor cultures and in clinical specimens. Int. J. Cancer, *54*, 1993, 268—274.

[24] ZÁVADA, J., ZÁVADOVÁ, Z., MACHOŇ, O., KUTINOVÁ, L., NĚMEČKOVÁ, Š., OPAVSKÝ, R., PASTOREK, J.: Transient transformation of mammalian cells by MN protein, a tumor-associated cell adhesion molecule with carbonic anhydrase activity. Int. J. Oncol., *10*, 1997, 857—863.